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(54) Title: CHEMICALLY-INDUCIBLE *ARABIDOPSIS* PR-1 PROMOTER

(57) Abstract

The nucleic acid sequence of the full-length, chemically inducible *Arabidopsis* PR-1 promoter has been discovered and is disclosed herein. Furthermore, *cis*-acting regulatory elements in the *Arabidopsis* PR-1 promoter involved in chemical induction have been characterized using deletion and linker-scanning mutagenesis and *in vivo* footprinting. It has been discovered that at least a portion of the region of promoter between positions -698 and -621 (relative to the transcription start site of the PR-1 gene) is required for induction of gene expression by chemicals. Two 10-bp linker-scanning mutations centered at 640-bp and 610-bp upstream from the transcription start site abolish the inducibility of the promoter while another 10-bp mutation centered at -670 bp results in average induced expression levels 4-fold higher than the unmutated promoter. Additionally, inducible *in vivo* footprints are located at positions -629 and -628 and at position -604 on the coding strand and at position -641 on the non-coding strand. The use of chemically inducible *Arabidopsis* PR-1 promoter fragments to regulate gene expression in plants in the presence of inducing chemicals such as SA, INA, and BTH is disclosed, as well as the use of these elements for the isolation of transcriptional regulatory proteins involved in the promoter regulation and for the construction of inducible hybrid promoters.

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CHEMICALLY-INDUCIBLE *ARABIDOPSIS* PR-1 PROMOTER

This application claims the benefit of U.S. Provisional Application No. 60/027,228, filed July 23, 1996, the disclosure of which is hereby expressly incorporated by reference in its entirety into the instant disclosure.

FIELD OF THE INVENTION

The invention generally relates to non-coding DNA sequences that regulate the transcription of associated DNA sequences in the presence of chemical regulators. The invention more particularly relates to the *Arabidopsis* PR-1 promoter, as well as deletion and linker-scanning mutants thereof, and its use in regulating gene expression in plants in the presence of chemical regulators. The invention further relates to sequences in the PR-1 promoter that are necessary for induction by chemicals and that are involved in its inducibility as well as their use for the isolation of transcriptional regulatory proteins and for the construction of inducible hybrid promoters.

BACKGROUND OF THE INVENTION

Advances in recombinant DNA technology coupled with advances in plant transformation and regeneration technology have made it possible to introduce new genetic material into plant cells, plants, or plant tissue. The target plants can range from trees and shrubs to ornamental flowers and field crops and even to cultures of plant tissue grown in bioreactors.

Regardless of the target of genetic engineering of plants, a principal advantage to be realized is the controlled expression of chimeric genes so that they are expressed only at the appropriate time, to the appropriate extent, and in some situations in particular parts of the plant. For example, the energy expended by a plant to continuously produce high levels of a foreign protein could prove detrimental to the plant, whereas if the gene were expressed only when desired, the drain on energy and therefore yield could be reduced. Additionally, the phenotype expressed by the chimeric gene could result in adverse effects to the plant if expressed at inappropriate times during development. For tissue in culture or in a bioreactor,

the untimely production of a desired secondary product could lead to a decrease in the growth rate of the culture, resulting in a decrease in the yield of the product.

In view of such considerations, it is apparent that control of the time, extent, and/or site of expression of chimeric genes in plants or plant tissues would be highly desirable. An ideal situation would be the at-will regulation of expression of an engineered trait via a regulating intermediate that could be easily applied to field crops, ornamental shrubs, bioreactors, etc. Such gene control could be of particularly great commercial value.

Several plant genes are known to be induced by various internal and external factors including plant hormones, heat shock, chemicals, pathogens, lack of oxygen, and light. For example, systemic acquired resistance (SAR) is a plant defense mechanism that occurs in diverse plant/pathogen interactions following a primary pathogen infection (Ryals, *et al.*, *Plant Cell* 8, 1809-1819 (1996), incorporated by reference herein in its entirety). It often leads to a hypersensitive response associated with the formation of necrotic lesions and to a substantial increase of the endogenous salicylic acid (SA) level. As a result, the plant becomes resistant to a variety of pathogens through a non-specific "immunization". This phenomenon is tighly correlated with the expression of several classes of genes, called pathogenesis-related (PR) genes (Ward, *et al.*, *Plant Cell* 3, 1085-1094 (1991), incorporated by reference herein in its entirety). Interestingly, exogenous application of SA also induces SAR and expression of PR genes (Ward, *et al.* 1991; Uknes, *et al.*, *Plant Cell* 4, 645-656 (1992), incorporated by reference herein in its entirety) as well as of synthetic compounds, such as 2,6-dichloroisonicotinic acid (INA) (Vernooij, *et al.*, *MPMI* 8, 228-234 (1995) incorporated by reference herein in its entirety) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Friedrich, *et al.*, *Plant J.* 10(1), 61-70 (1996), incorporated by reference herein in its entirety; Lawton, *et al.*, *Plant J.* 10, 71-82 (1996), incorporated by reference herein in its entirety). Therefore, induction of PR protein genes by chemicals or pathogens provides a foundation to address the problem of controlling gene expression in plants and plant tissue.

Several steps in the signal transduction leading to the onset of SAR have been elucidated. Overexpression of a bacterial salicylate hydroxylase gene (nahG) has been shown to suppress SAR, indicating that SA accumulation is required for its onset (Gaffney, *et al.*, *Science* 261, 754-756 (1993), incorporated by reference herein in its entirety). Recently, SAR has been characterised in *Arabidopsis* and the corresponding PR-1, PR-2 and PR-5 genes

have been isolated (Uknes, *et al.* 1992), thus facilitating the isolation of mutants with altered SAR. Mutants with constitutive SAR such as cpr1 (Bowling, *et al.*, *Plant Cell* 6, 1845-1857 (1994)) have been described, as well as mutants defective in SAR such as npr1 (Cao, *et al.* 1994) and nim1 (Delaney, *et al.*, *Proc. Nat. Acad. Sci.* 92, 6602-6606 (1995), incorporated by reference herein in its entirety). In the case of nim1, a mutagenised population of *Arabidopsis* was screened for lack of PR-1 gene expression after pathogen treatment (Delaney, *et al.* 1995). NIM1 and NPR1 were subsequently shown to include mutations in the same open reading frame (Cao, *et al.* 1997; Ryals, *et al.*, *Plant Cell* 9, 425-439 (1997), incorporated by reference herein in its entirety) that coded for a protein with extensive homology to the transcriptional repressor I-KB (Ryals, *et al.* 1997).

Studies of the effects of chemical regulators on the promoters of several PR genes have also been described in the literature, shedding additional light of the SAR signal transduction pathway. Deletion analysis of the tobacco PR-1a promoter revealed that a 600-bp long promoter had lost its functional inducibility by pathogen infection as well by exogenous chemical application while 661-bp retained inducibility although to a lesser extent than a 903-bp long fragment (Uknes, *et al.*, *Mol. Plant-Microbe Interact.* 6, 692-698 (1993), incorporated by reference herein in its entirety). Analysis of the tobacco PR-2d promoter revealed that some of its inducility is lost in a 607-bp long fragment but that 1047-bp were required for maximal induction, while a 321-bp long promoter had lost almost any inducibility (Hennig, *et al.* 1993). More recently, a Myb-like transcrition factor (myb1) was isolated and its expression shown to be inducible by SA and tobacco mosaic virus (Yang, *et al.* 1996). Furthermore it was shown to bind *in vitro* to a fragment of the tobacco PR-1a promoter (positions -679 to -487 from the transcription start site) containing a Myb-like recognition site (positions -520 to -514). Moreover, a sequence in the tobacco PR-2d promoter (-348 to -324) was shown to bind *in vitro* to a yet unidentified protein. Mutation of this sequence reduced inducibility by SA by approximately 3-fold but not completely compared to wild-type sequence when included in a fragment spanning positions -364 to -288 in the PR-2d promoter and fused to the -90 35S CaMV promoter (Shah, *et al.* 1996).

United States Patent No. 5,614,395, incorporated by reference herein in its entirety, describes the *Arabidopsis* PR-1 protein gene and its chemically inducible promoter. As described in this patent, the cDNA sequence encoding this PR protein was cloned into plasmid pAPR1C-1, ATCC number 75049, deposited July 12, 1991. In addition, it was

disclosed that the full-length promoter fragment (4.2 kb) from the PR-1 coding sequence was isolated and cloned into plasmid pAtPR1-P, which was deposited January 5, 1994, with the Agricultural Research Culture Collection, International Depositing Authority and assigned NRRL number NRRL B-21169. It was further disclosed in this patent that the full-length *Arabidopsis* PR-1 promoter fragment was fused to the firefly luciferase (LUC) gene and ultimately cloned into plasmid pAtPR1-S, which was in turn transformed into *Arabidopsis* plants for chemical induction analysis. The transgenic *Arabidopsis* lines carrying the PR-1 promoter/LUC gene fusion were then treated by spraying with isonicotinic acid (INA). When analyzed, the transgenic lines showed significantly higher induction of luciferase activity compared to water-treated controls. Thus, INA was shown to induce expression in transformed plants of a chimeric gene comprising the full-length *Arabidopsis* PR-1 promoter fragment.

As described in U.S. Patent No. 5,614,395, only a PR-1 promoter/LUC gene fusion comprising the full-length (4.2 kb) PR-1 promoter was analyzed. In addition, the nucleotide sequence of the *Arabidopsis* PR-1 promoter was not disclosed. However, it is often the case that smaller DNA fragments are easier to manipulate than larger DNA fragments. In this instance, by reducing the PR-1 promoter sequence to its minimal essential portion, creating a smaller DNA fragment, chimeric constructs comprising the minimal promoter fragment joined to a coding sequence could in certain situations be more easily made and utilized than constructs comprising a full-length promoter fragment.

Therefore, one object of the instant invention is to determine the nucleotide sequence of the chemically regulatable *Arabidopsis* PR-1 promoter. Based on this knowledge, additional objects of the instant invention are to determine the minimal length of the *Arabidopsis* PR-1 promoter required for chemical induction by using deletion mutagenesis and to characterize *cis*-acting regulatory elements in the *Arabidopsis* PR-1 promoter involved in chemical induction by using linker-scanning mutagenesis and in-vivo footprinting.

SUMMARY OF THE INVENTION

The present invention provides the nucleic acid sequence of the full-length *Arabidopsis* PR-1 promoter, which is shown in SEQ ID NO: 1. In addition, the present invention encompasses chimeric genes comprising the *Arabidopsis* PR-1 promoter

operatively linked to a coding sequence of a gene of interest, wherein the *Arabidopsis* PR-1 promoter regulates transcription of the coding sequence in the presence of chemical regulators. In a preferred embodiment, the coding sequence encodes an assayable marker, such as an enzyme, whereby expression of the enzyme can be observed in assays for chemical induction of the chimeric gene. In related aspects, the present invention also embodies a recombinant vector, such as a plasmid, comprising the aforementioned chimeric gene, as well as a plant or plant tissue stably transformed with such a vector.

Another aspect of the present invention relates to the discovery that a certain region of the *Arabidopsis* PR-1 promoter is required for chemical regulation. In particular, a region of the *Arabidopsis* PR-1 promoter between 698-bp and 621-bp upstream from the transcription start site of the PR-1 gene is necessary for induction of gene expression by chemicals such as salicylic acid (SA) compounds, isonicotinic acid (INA) compounds, and benzo-1,2,3-thiadiazoles (BTH). Inducibility of a 698-bp long promoter fragment is reduced by approximately 2-fold compared to longer promoter fragments and inducibility of promoter fragments of 621-bp or shorter is lost altogether. Therefore, the present invention further embodies deletion mutants that are shorter than the full-length 4,258-bp *Arabidopsis* PR-1 promoter sequence, yet still yield similar induction of gene expression upon the application of a chemical regulator. These deletion mutants may be used to form chimeric genes, which in turn may be cloned into vectors and transformed into plants in the same manner as the full-length sequence.

A further aspect of the present invention relates to the discovery via linker-scanning mutagenesis that two 10-bp mutations centered at 640-bp and 610-bp upstream from the transcription start site (+1) abolish the inducibility of the promoter while another 10-bp mutation centered at 670-bp upstream from the transcription start site results in induced expression levels 4-fold higher than the unmutated promoter. The 640-bp linker-scanning mutation encompasses a recognition site for transcription factors of the basic leucine zipper class, such as CREB, while the 610-bp linker-scanning mutation contains a sequence similar to a recognition site for the transcription factor NF-kB. Furthermore, inducible *in-vivo* footprints are located at positions -629 and -628 and at position -604 on the coding strand and at position -641 on the non-coding strand, indicating that this region of the promoter undergoes changes in protein/DNA interactions upon chemical induction.

The invention therefore describes important regulatory elements involved in the chemical induction of the PR-1 promoter. These elements can be used for the isolation of transcriptional regulatory proteins involved in the promoter regulation and for the construction of inducible hybrid promoters. These elements can further be used as probes for other chemically inducible promoters from *Arabidopsis* as well as chemically inducible promoters from other plants.

BRIEF DESCRIPTION OF THE FIGURE

FIG. 1 depicts the structure of linker-scanning mutants LS1-LS13. The wt sequence is taken from SEQ ID NO: 1. Nucleotides that are actually changed are shown in bold.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO: 1 - Nucleic acid sequence of the full-length (4,258-bp long) *Arabidopsis thaliana* PR-1 promoter (in plasmid pLTD1D). Also shown are the nucleic acid sequence of the 5' untranslated region as well as the start of the coding region for the PR-1 gene. Nucleotides 3444 - 4258 constitute the 815-bp long *Arabidopsis thaliana* PR-1 promoter fragment (in plasmid pLTD7D) that confers essentially the same levels of chemical induction of gene expression as the full-length (4,258-bp long) promoter. Nucleotides 3561 - 4258 constitute the 698-bp long *Arabidopsis thaliana* PR-1 promoter fragment (in plasmid pLTD71D) that also confers chemical induction of gene expression, although to levels reduced by approximately 3-4 fold as compared to the full-length promoter. Nucleotides 3638 - 4258 constitute the 621-bp long *Arabidopsis thaliana* PR-1 promoter fragment (in plasmid pLTD72D) that confers no chemical induction of gene expression.

SEQ ID NO: 2 - primer ext1.

SEQ ID NO: 3 - primer 1a.

SEQ ID NO: 4 - primer 1b.

SEQ ID NO: 5 - primer 1c.

SEQ ID NO: 6 - primer 1d.

SEQ ID NO: 7 - primer N1,076.

SEQ ID NO: 8 - primer PR1R.

SEQ ID NO: 9 - primer N781.

SEQ ID NO: 10 - primer N704.

SEQ ID NO: 11 - primer LS1-.

SEQ ID NO: 12 - primer LS1+.

SEQ ID NO: 13 - primer LS2-.

SEQ ID NO: 14 - primer LS3-.

SEQ ID NO: 15 - primer LS4-.

SEQ ID NO: 16 - primer LS5-.

SEQ ID NO: 17 - primer LS6-.

SEQ ID NO: 18 - primer LS7-.

SEQ ID NO: 19 - primer LS8-.

SEQ ID NO: 20 - primer LS9-.

SEQ ID NO: 21 - primer LS2+.

SEQ ID NO: 22 - primer LS3+.

SEQ ID NO: 23 - primer LS4+.

SEQ ID NO: 24 - primer LS5+.

SEQ ID NO: 25 - primer LS6+.

SEQ ID NO: 26 - primer LS7+.

SEQ ID NO: 27 - primer LS8+.

SEQ ID NO: 28 - primer LS9+.

SEQ ID NO: 29 - primer LS10-.

SEQ ID NO: 30 - primer LS11-.

SEQ ID NO: 31 - primer LS12-.

SEQ ID NO: 32 - primer LS102.

SEQ ID NO: 33 - primer LS103.

SEQ ID NO: 34 - primer LS112.

SEQ ID NO: 35 - primer LS113.

SEQ ID NO: 36 - primer LS122.

SEQ ID NO: 37 - primer LS123.

SEQ ID NO: 38 - primer LS13-.

SEQ ID NO: 39 - primer P1-

SEQ ID NO: 40 - primer LMPCR2

SEQ ID NO: 41 - primer LMPCR3

SEQ ID NO: 42 - primer P2-

SEQ ID NO: 43 - primer P3-

SEQ ID NO: 44 - primer P41+

SEQ ID NO: 45 - primer LMPCR1

SEQ ID NO: 46 - primer P52+

SEQ ID NO: 47 - primer P53+

SEQ ID NO: 48 - recognition site of the yeast transcription factor GCN4

SEQ ID NO: 49 - recognition site of the bZIP transcription factor CREB

SEQ ID NO: 50 - recognition site for bZIP transcription factors

SEQ ID NO: 51 - consensus recognition sequence of NF-kB

DETAILED DESCRIPTION OF THE INVENTION

The present invention encompasses various aspects of the *Arabidopsis* PR-1 promoter, including the discovery that a particular region is required for its induction by chemicals. The full-length *Arabidopsis thaliana* PR-1 promoter was originally isolated as a fragment having a length of 4,258-bp relative to the transcription start site of the PR-1 gene. The nucleic acid sequence of the full-length promoter has been discovered and is shown in SEQ ID NO: 1.

In addition, it has been discovered that at least a portion of the region of the *Arabidopsis thaliana* PR-1 promoter located between 698-bp and 621-bp upstream from the transcription start site (between nucleotides 3561 and 3638 of SEQ ID NO: 1) is required for chemical induction of gene expression. The present invention therefore encompasses an isolated DNA molecule that constitutes the full-length PR-1 promoter sequence, as well as isolated DNA molecules that constitute relatively minimal PR-1 promoter sequences but still include the necessary region between nucleotides 3561 and 3638. These promoter sequences can be operatively linked to a coding sequence to form a chimeric gene, whereupon the promoter sequence will regulate transcription of the coding sequence. The chimeric gene can be cloned into a recombinant vector, which can then in turn be stably transformed into a host. The transformed host will then exhibit expression of the chimeric gene upon treatment with a chemical regulator. Thus, the present invention also encompasses chimeric genes comprising either the full-length PR-1 promoter or one of the chemically inducible PR-1 promoter

fragments operatively linked to a coding sequence; recombinant vectors comprising one of these chimeric genes; and host plants transformed with one these vectors.

The coding sequence forming a component of the chimeric gene comprises any transcribable DNA sequence such that the chimeric gene is capable of being expressed in a host under the proper conditions of chemical regulation. The coding sequence may be derived from natural sources or be prepared synthetically. In one embodiment, the coding sequence may be transcribed as an RNA that is capable of regulating the expression of a phenotypic trait by an anti-sense mechanism. Alternatively, the coding sequence in the chimeric gene may be transcribed and translated, i.e. coded, in the plant tissue to produce a polypeptide that imparts a phenotypic trait to the host. For example, a chimeric gene designed to be transformed into a host plant could comprise a coding sequence that encodes one of the following: a gene controlling flowering or fruit ripening; a gene effecting tolerance or resistance to herbicides (i.e., a gene coding for wild-type or herbicide resistant acetohydroxyacid synthase (AHAS)) or to many types of pests, for example fungi, viruses, bacteria, arachnids, nematodes, or insects (i.e., a gene coding for *Bacillus thuringiensis* endotoxin (BT)); a gene controlling production of enzymes or secondary metabolites; or a gene confering male or female sterility, dwarfness, flavor, nutritional qualities, or the like.

In a preferred embodiment, the coding sequence encodes an enzyme, such as an assayable marker, whereby expression of the enzyme can be observed in assays for chemical induction of the chimeric gene. Suitable assayable markers that may be encoded by the coding sequence include, but are not limited to, the following: luciferase (LUC), chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), and β-glucuronidase (GUS). An especially preferred marker is β-glucuronidase (GUS). Chimeric genes comprising one of these assayable markers are particularly useful because the effect of the chemical induction, i.e. beta-glucuronidase enzyme activity, is easily detectable in plant cells or extracts thereof.

Recombinant vectors, produced by standard techniques but comprising the chimeric genes described above, represent an additional feature of the invention. Vectors are recombinant DNA sequences that may be used for isolation and multiplication purposes of the mentioned DNA sequence and for the transformation of suitable hosts with these sequences. Preferred vectors for isolation and multiplication are plasmids that can be propagated in a suitable host microorganism, for example in *E. coli*. Preferred vectors for

transformation are those useful for transformation of plant cells or of *Agrobacteria*. For *Agrobacterium*-mediated transformation, the preferred vector is a Ti-plasmid derived vector. For direct DNA transfer into protoplasts, any of the aforementioned vectors may be used.

Various chemical regulators may be employed to induce expression of the coding sequence in the chimeric genes constructed according to the present invention. In the context of the instant disclosure, "chemical regulators" include chemicals known to be inducers for PR proteins in plants, or close derivatives thereof. These include benzoic acid, salicylic acid (SA), polyacrylic acid and substituted derivatives thereof; suitable substituents include lower alkyl, lower alkoxy, lower alkylthio, and halogen. An additional group of regulators for the chemically inducible promoters sequences and chimeric genes of this invention is based on the benzo-1,2,3-thiadiazole (BTH) structure and includes, but is not limited to, the following types of compounds: benzo-1,2,3-thiadiazolecarboxylic acid, benzo-1,2,3-thiadiazolethiocarboxylic acid, cyanobenzo-1,2,3-thiadiazole, benzo-1,2,3-thiadiazolecarboxylic acid amide, benzo-1,2,3-thiadiazolecarboxylic acid hydrazide, benzo-1,2,3-thiadiazole-7-carboxylic acid, benzo-1,2,3-thiadiazole-7-thiocarboxylic acid, 7-cyano-benzo-1,2,3-thiadiazole, benzo-1,2,3-thiadiazole-7-carboxylic acid amide, benzo-1,2,3-thiadiazole-7-carboxylic acid hydrazide, alkyl benzo-1,2,3-thiadiazolecarboxylate in which the alkyl group contains one to six carbon atoms, methyl benzo-1,2,3-thiadiazole-7-carboxylate, n-propyl benzo-1,2,3-thiadiazole-7-carboxylate, benzyl benzo-1,2,3-thiadiazole-7-carboxylate, benzo-1,2,3-thiadiazole-7-carboxylic acid sec-butylhydrazide, and suitable derivatives thereof. Still another group of regulators for the chemically inducible DNA sequences of this invention is based on the pyridine carboxylic acid structure, such as the isonicotinic acid structure and preferably the haloisonicotinic acid structure. Preferred are dichloroisonicotinic acids and derivatives thereof, for example the lower alkyl esters. Suitable regulators of this class of compounds are, for example, 2,6-dichloroisonicotinic acid (INA), and the lower alkyl esters thereof, especially the methyl ester.

The chimeric genes constructed according to the present invention may be transformed into any suitable host cell; however, the chimeric genes are preferably transformed into plant tissue. As used in conjunction with the present invention, the term "plant tissue" includes, but is not limited to, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell cultures, and any groups of plant cells organized into structural and/or functional units. Plants transformed with the chimeric genes of the present invention

may be monocots or dicots and include, but are not limited to, maize, wheat, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato,sorghum, sugarcane, sugarbeet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, and Arabidopsis.

The chimeric genes of the instant invention and vectors containing these genes can be introduced into plant cells by a variety of techniques that give rise to transformed cells, tissue, and plants or to cell cultures useful in bioreactors. Several techniques are described in detail in the examples that follow. Other methods included here for enabling purposes, which are directed to both monocots and dicots, are disclosed in U.S. Patent No. 5,614,395. Such methods used for transfer of DNA into plant cells include, for example, the direct infection of or co-cultivation of plants, plant tissue, or cells with *Agrobacterium tumefaciens* (Horsch, R.B. *et al., Science 225:* 1229 (1985); Marton, L., *Cell Culture and Somatic Cell Genetics of Plants 1:* 514-521, 1984). Additional methods include direct DNA uptake, liposomes, electroporation, micro-injection, and microprojectiles. Such methods have been published in the art. See, for example, Bilang, *et al., Gene* 100: 247-250 (1991); Scheid *et al., Mol. Gen. Genet.* 228: 104-112 (1991); Guerche *et al., Plant Science* 52: 111-116 (1987); Neuhause *et al., Theor. Appl. Genet.* 75: 30-36 (1987); Klein *et al., Nature* 327: 70-73 (1987); Howell *et al., Science* 208: 1265 (1980); Horsch *et al., Science* 227: 1229-1231 (1985); DeBlock *et al., Plant Physiology* 91: 694-701 (1989); *Methods for Plant Molecular Biology* (Weissbach and Weissbach, eds.) Academic Press Inc. (1988); and *Methods in Plant Molecular Biology* (Schuler and Zielinski, eds.) Academic Press Inc. (1989). See also, U.S. Patent Nos. 4,945,050; 5,036,006; and 5,100,792, all to Sanford *et al.* In addition, see U.S. Patent Application Serial Nos. 08/438,666, filed May 10, 1995, and 07/951,715, filed Sept. 25, 1992, both of which are hereby incorporated by reference in their entireties.

Practical applications of this invention include the controlled expression of chimeric genes in crop plants so that they are expressed only at the appropriate time, to the appropriate extent, and/or in particular parts of the plant. For example, the effectiveness of disease resistance or insect resistance in transgenic plants transformed with genes encoding disease-resistant or insect-resistant proteins, respectively, could be enhanced if the timing of the

expression could be controlled. See, e.g., Uknes, *Plant Cell, 4:* 645-656 (1992); Ward *et al., Plant Cell 3:*1085-1094 (1991); Gould, *Bioscience 38:* 26-33 (1988); and Gould, *TIBTECH 6:* S15-S18 (1988). Also, the chemical regulation of developmental processes such as homeosis, germination, tillering, sprouting, flowering, anthesis, fruit ripening, and abscission offers several advantages such as the facilitated production of hybrid seed, greater reduction of crop loss, and more generally, control of the growth and development of the plant by the farmer. Thus, the present invention applies equally to transgenic plants containing heterologous genes, e.g., disease resistance genes including PR and SAR genes, insect resistance genes such as BT genes, and genes involved in developmental processes such as those described above. It also includes genes encoding industrial or pharmaceutical biomaterials such as plastics and precursors thereof, perfumes, additives, enzymes and other proteins, and pharmaceuticals, wherein the plant effectively would be used as a bioreactor, e.g., the two genes encoding production of polyhydroxybutyrate, a thermoplastic (Poirier *et al., Science 256:* 520-523 (1992).

As described more fully below in the examples, the full-length PR-1 promoter sequence was fused to the β-glucuronidase (GUS) gene at the native ATG to obtain a chimeric gene cloned into plasmid pLTD1D. Plasmid pLTD1D was then digested with restriction enzymes to release a fragment comprising the full-length PR-1 promoter sequence and the GUS gene, which was then used to construct the binary vector designated pCIB/LTD1D. This binary vector was transformed into *Agrobacterium tumefaciens*, which was in turn used to transform Arabidopsis plants.

Plasmid pLTD1D was also used to form a series of 5' end deletion mutants having increasingly shorter PR-1 promoter fragments fused to the GUS gene at the native ATG. Various restriction enzymes were used to digest plasmid pLTD1D to obtain the binary vectors with different lengths of promoter fragments. In particular, pLTD5D was constructed with a 1,974-bp long promoter fragment; pLTD6D was constructed with a 1,293-bp long promoter fragment; pLTD61D was constructed with a 984-bp long promoter fragment; pLTD7D was constructed with a 815-bp long promoter fragment; pLTD71D was constructed with a 698-bp long promoter fragment; pLTD72D was constructed with a 621-bp long promoter fragment; pLTD8D was constructed with a 572-bp long promoter fragment; and pLTD9D was constructed with a 78-bp long promoter fragment. Like the binary vector comprising the full-

length PR-1 promoter fragment, these 5' end deletion mutants were also transformed into *Agrobacterium tumefaciens* and, in turn, Arabidopsis plants.

Each of the transgenic Arabidopsis lines was treated by spraying with isonicotinic acid (INA), a known inducer of the PR-1 promoter. Green tissue was harvested three days after treatment and subjected to a GUS enzyme assay to determine the amount of protein expressed as a result of induction of each chimeric gene. For each transgenic line, the induction of GUS expression by INA was obtained by dividing the specific activity of the INA-treated sample by the specific activity of an untreated control sample.

As expected, upon treatment with INA, plants transformed with the chimeric gene including the full-length (4,258-bp long) PR-1 promoter demonstrated greatly increased induction of GUS expression compared to controls. In addition, the 1,974-bp, 1,293-bp, 984-bp, and 815-bp long promoter fragments yielded similar induction of GUS expression by INA. The 698-bp long promoter fragment still yielded high inducibility by INA, although reduced by approximately 3-4 fold compared to the longer promoter fragments. However, the 621-bp, 572-bp, and 78-bp long promoter fragments yielded substantially no induction of GUS expression by INA. These results are shown in Table 1, which presents the average values of GUS activity in pmole MU/mg protein/min (INA-treated/untreated controls) for the transgenic lines containing the PR-1 promoter constructs.

The presence of the correct hybrid construct in the transgenic lines was confirmed by PCR amplification. INA induction of the endogenous PR-1 gene was confirmed by Northern blot analysis for transgenic lines containing the 1,293-bp, 689-bp, 621-bp, 574-bp and 78-bp long constructs (5 lines per construct) indicating that the loss of inducibility of GUS expression was due to the gene construct and not to lack of SAR-mediated induction of gene expression in these particular lines or samples.

Thus, at least a portion of the region of the *Arabidopsis thaliana* PR-1 promoter located between positions 3561 and 3638 of SEQ ID NO: 1 (between 698-bp and 621-bp upstream from the transcription start site) is required for chemical induction of gene expression. Its removal completely abolishes PR-1 promoter induction. Moreover, additional elements located between positions -815 and -698 also contribute to full inducibility of the promoter. Minimal PR-1 promoter fragments having lengths substantially less than the full-length PR-1 promoter can therefore be operatively linked to coding sequences to form smaller constructs than can be formed using the full-length PR-1 promoter. As noted earlier, shorter

DNA fragments are often more amenable to manipulation than longer fragments. The chimeric gene constructs thus formed can then be transformed into hosts such as crop plants to enable at-will regulation of coding sequences in the hosts.

While a deletion analysis characterizes regions in a promoter that are required overall for its regulation, linker-scanning mutagenesis allows for the identification of short defined motifs whose mutation alters the promoter activity. Accordingly, a set of 13 linker-scanning mutant promoters fused to the coding sequence of the GUS reporter gene (LS1 to LS13, Figure 1) was constructed. Each of them contained a 10-bp mutation (8-bp for LS12) located between positions -705 and -578 (nucleotides 3554 to 3681 of SEQ ID NO: 1) and included in a 1,293-bp long promoter fragment (nucleotides 2966 to 4258 of SEQ ID NO: 1). Each construct was transformed into *Arabidopsis* and GUS activity was assayed for 19 to 30 independent transgenic lines. The presence of the correct hybrid construct in transgenic lines was confirmed by PCR amplification of all lines containing LS7 and LS10 constructs and by random sampling of lines containing the other constructs. Amplified fragments were digested with XbaI and separated on high resolution agarose gels to distinguish between the different LS constructs. The effect of each mutation on promoter activity was compared to an equivalent number of transgenic lines containing the unmutated 1,293-bp construct. Two repetitions resulting from independent plating of seeds and INA-treatments were carried out in every case.

Most LS mutations had no effect or minor effects on the promoter activity. However, 3 of them had dramatic effects on the promoter function. One construct, LS4 (introducing a mutation at positions -666 to -675 (nucleotides 3584 to 3593 of SEQ ID NO: 1)), resulted in 2-fold higher average induction of GUS activity by INA than the control construct and approximatively 4-fold and 3-fold higher average GUS activity for INA- and water-treated samples, respectively (Table 2). This suggested that a negative regulatory element had been mutated in this construct or, less likely, that a sequence responsible for higher promoter activity had been introduced. Since both induced and uninduced expression levels were influenced, the mutation seemed to affect a constitutive negative regulatory element. Interestingly, LS5 which contained a mutation adjacent to the mutation in LS4, also led to 3-fold higher average levels of GUS activity after water treatment but INA-induced levels of activity for LS5 were similar to the control construct. This suggested that such a regulatory element may span the sequences affected by both mutations.

In two constructs, LS7 (mutation at positions -636 to -645 (nucleotides 3614 to 3623 of SEQ ID NO: 1)) and LS10 (mutation at positions -606 to -615 (nucleotides 3644 to 3653 of SEQ ID NO: 1)), a complete loss of inducibility of GUS activity by INA was observed. Average GUS values for both water and INA treatments in transgenic lines containing LS7 were similar to water-treated values for lines containing the control construct. In the case of LS10, average GUS values were slightly higher because of two lines showing high uninduced and induced GUS activity. These results are consistent with the presence of a positive regulatory element that is necessary for induction of PR-1 gene expression by INA in or near the LS7 and LS10 locations.

INA-induction of the endogenous PR-1 gene was monitored by Northern blot analysis for transgenic lines containing LS1, LS4, LS7 and LS10 (5 lines per construct) and did not significantly differ from lines containing the control construct, indicating that the loss of inducibility of GUS expresion was due to the gene construct and not to lack of or higher SAR-mediated induction of gene expression in these particular lines or samples.

The sequences mutated in the linker-scanning constructs, in particular those that showed marked differences from the control construct, were examined more closely. For LS4, a perfect homology was found to the recognition site of the yeast transcription factor GCN4 ("TGACTG" (SEQ ID NO: 48)), a member of the basic leucine zipper (bZIP) family. The sequence mutated in LS5 contained a perfect homology to the recognition site of CREB ("CTACGTCA" (SEQ ID NO: 49)), a member of the bZIP transcription factor family as well.

Mutations in LS7 and LS10 that had the most dramatic effects on the promoter activity also contained interesting sequences. A recognition site for bZIP transciption factors ("ACGTCA" (SEQ ID NO: 50)) was found in LS7 and a sequence similar to the binding site of transcription factors of the Rel family, such as NF-kB, was found in LS10 ("GGACTTTTC" compared to the consensus recognition sequence "GGGACTTTTCC" (SEQ ID NO: 51)). No significant homology to binding sites of known transcription factors could be found in the sequences mutated in the remaining linker-scanning constructs.

SA is an exogenous signal for gene expression that can also be applied exogenously. Although all data suggest that INA and SA act on plant analogously, experiments were conducted to determine whether the effects of the two compounds on the linker-scanning promoters were identical. For each linker-scanning construct, five lines showing medium to strong inducibility by INA were treated in parallel with water, 0.325mM INA, and 5mM SA.

The responses of the different constructs to SA and INA were similar when normalized to the effect of each compound on the control construct. This observation supports the commonly held assumption that INA and SA act through the same pathway for induction of expression of PR-genes. Induction of LS4 by SA was approximately 2-fold higher than induction of the control construct by SA, as observed before with INA. For the five lines containing LS7, an average 1.5-fold induction by SA was measured compared to 1.4-fold by INA (in each case as an average of two independent repetitions). Interestingly, an average 4.0-fold induction by SA was measured for LS10 compared to 1.6-fold by INA. This suggests that the effect of the mutation in LS10 was less dramatic than in LS7 and that this difference could only be detected under stronger inductive conditions such as the treatment with SA.

Organ-specific GUS expression was examined in roots and floral tissues of three independent untreated lines per linker-scanning construct. In the control construct, some weak GUS expression was detected in male organs but no expression could reproducibly be detected in other flowers parts or roots. In the linker-scanning constructs a similar pattern of GUS expression was observed indicating that the mutations did not dramatically affect organ-specific expression of the PR-1 promoter. However, for some constructs, the intensity of GUS expression in the male floral organs differed from the control construct. In the three lines containing LS4 and LS5, expression was higher whereas in the three lines containing LS7 and LS10, almost no GUS activity was detected. Therefore, the mutations appear to have similar up and down regulating effects on the promoter activity in uninduced male floral organs as well as in uninduced green tissue.

In-vivo footprinting is based on methylation of guanine bases at position N7 by dimethylsulfate (DMS) followed by specific cleavage of the methylated guanines by piperidine. Changes in DNA occupancy by DNA-binding proteins alter the accessibility of DNA by the methylating agent, thereby yielding changes in populations of cleaved molecules after piperidine treatment. After LM-PCR a "G" ladder is resolved on a sequencing gel and differences in intensities of specific bands can be related to differences in DNA protection at the particular guanines.

Analysis of the coding strand revealed inducible footprints at positions -629 and -628 and at position -604 (FIG.1) (nucleotides 3630, 3631, and 3655 of SEQ ID NO: 1, respectively). At both sites, an increased band intensity after INA treatment was detected, indicating deprotection of these guanines upon INA treatment. These two footprints are

located in sequences mutated by linker-scanning constructs LS8 and LS11 (FIG. 1). Neither construct seemed to significantly influence the regulation of the promoter, but they are surrounding the sequence mutated in construct LS10, which had lost inducibility by INA. This suggests that that the region of the PR-1 promoter around this site undergoes changes in its occupancy by DNA-binding proteins. No other change in band intensities could be detected in the range of DMS concentrations tested (0.04% to 1%), suggesting that the described sites are the only ones affected after INA treatment.

Examination of the non-coding strand revealed an inducible footprint at position -641 (3618 of SEQ ID NO: 1). In this case, too, deprotection was observed. The guanine at position -641 is included in the sequence mutated in linker-scanning mutant LS7, which had lost inducibility by INA and that contains a recognition site for basic leucine zipper transcription factors at positions -645 to -640. Different DMS concentrations did not reveal any other inducible footprint.

These results show that the examined region undergoes changes in protein-DNA interactions and suggest that the above described elements are required for the induction itself ("switch" element) and are not just binding sites for constitutive transcriptional activators.

EXAMPLES

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory manual, Cold Spring Harbor laboratory, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. *et al.*, Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

A. Full-Length *Arabidopsis* PR-1 Promoter

Example 1: Determination of the Transcription Start Site of the *Arabidopsis* PR-1 gene

RNA was purified from frozen tissue by phenol/chloroform extraction followed by lithium chloride precipitation (Lagrimini et al. (1987), Proc. Natl. Acad. Sci. USA 84: 7542-7546). A PR-1 specific right-to-left "bottomstrand" oligonucleotide corresponding to positions +59 to +32 downstream from the PR-1 ATG (ext1: AAG AGC ACC TAC AAG AGC TAC AAA GAC G) (SEQ ID NO: 2) was labelled at its 5' end (Sambrook J. et al. (1989), Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York) and served as primer for the extension reaction carried out with AMV reverse transcriptase according to the manufacturer's recommendations (Promega, Madison, WI). The extension product was separated on a 6% polyacryamide gel and its length was determined by comparison with a sequence ladder obtained with pAtPR1-R (U.S. Patent No. 5,614,395) as a template and oligonucleotide ext1 as a primer using the Sequenase Version 2.0 DNA sequencing kit according to the manufacturer's conditions (USB, Cleveland, OH).

Example 2: Fusion of the *Arabidopsis* Full-Length PR-1 Promoter to the Coding Sequence of the β-glucuronidase (GUS) Gene

Plasmid pAtPR1-R was used as a template in PCR with a left-to-right "topstrand" primer extending from positions -268 to -251 upstream of the PR-1 ATG (primer 1a: GGC AAA GCT ACC GAT AC) (SEQ ID NO: 3) and a right-to-left "bottomstrand" primer comprising 11 bp of GUS coding sequence extending up to the GUS ATG and a further 9 bp of PR-1 sequence extending from the ATG into the PR-1 untranslated leader (primer 1b: GGA CGT AAC ATT TTT CTA AG) (SEQ ID NO: 4). This PCR reaction was undertaken with AmpliTaq DNA polymerase according to the manufacturer's recommendations (Perkin Elmer/Roche, Branchburg, NJ) for five cycles at 94°C (30 s), 40°C (60 s), and 72°C (30 s) followed by 25 cycles at 94°C (30 s), 55°C (60 s) and 72°C (30 s). This generated a product of 278 bp through annealing of the homologous PR-1 sequences; the fragment included a BglII site at its left end from the PR-1 promoter. A second PCR reaction was done using plasmid pBS-GUS1.2 (Uknes *et al.*, *The Plant Cell* 5:159-169 (1993)) as a template and using a left-to-right "topstrand" oligonucleotide, which comprised 9 bp of PR-1 untranslated leader up to the PR-1 ATG and a further 11 bp of GUS sequence from the ATG into the GUS coding sequence (primer 1c: CTT AGA AAA ATG TTA CGT CC) (SEQ ID NO: 5) and a right-to-left "bottom strand" oligonucleotide extending from positions -502 to -518 upstream of the PR-1 ATG into the GUS coding sequence (primer 1d: TTA CGC TGC GAT GGA TC) (SEQ ID NO: 6). This PCR reaction was done under the same conditions as the one described above and generated a fragment of 527 bp through annealing of the homologous GUS sequences; this fragment included a *Sna*BI site at its right end derived from the amplified GUS sequence.

The two PCR fragments generated above were gel purified using standard procedures to remove oligonucleotides and were then themselves mixed in a further PCR reaction ("inside-outside PCR") with primers 1a and 1d. Conditions for this reaction were the same as described above. The amplified fragment was a fusion of the PR-1 promoter fragment from the first PCR reaction described above and the GUS 5' coding sequence from the second PCR reaction described above and had a *Bgl*II site at its left end and a *Sna*BI site at its right end. The fragment was gel purified and cleaved with *Bgl*II and *Sna*BI (all restriction enzymes were purchased from Promega, Madison, WI) to yield a product of 497 bp in size that was ligated

in a three-way ligation with a BglII-SacI fragment of pAtPR1-R containing PR-1 promoter sequences upstream from the BglII site and a SnaBI-SacI fragment of pBSGUS1.2 containing the 3' end of GUS to obtain pLTD1D.

B. Deletion Mutant Analysis

Example 3: Preparation of Chimeric Genes Containing Variable Lengths of the PR-1 Promoter Sequence Fused to GUS

A. Construction of pLTD5D

Plasmid pLTD1D was digested with restriction enzymes *Xho*I and *Hpa*I; the protruding ends of the *Xho*I site were filled-in with Klenow DNA polymerase (Promega, Madison, WI); and the resulting blunt-ended vector fragment containing a 1,974-bp long promoter fragment was self-ligated.

B. Construction of pLTD6D

Plasmid pLTD1D was digested with restriction enzymes *Xho*I and *Sna*BI; the protruding ends of the *Xho*I site were filled-in with Klenow DNA polymerase; and the vector fragment containing a 1,293-bp long promoter fragment was self-ligated.

C. Construction of pLTD61D

Plasmid pLTD1D was used as template for PCR with a left-to-right "topstrand" primer comprising a *Xho*I restriction site and extending from position -1,019 to -1,000 upstream of the PR-1 ATG (primer N1,076: ACC GCT CGA GAA TTT TTC TGA TTC GGA GGG) (SEQ ID NO: 7) and a right-to-left "bottomstrand" primer extending from position -584 to -607 upstream of the PR-1 ATG (primer PR1R: TAT TTG TTT CTT AGT GTT TCA TGC) (SEQ ID NO: 8). The PCR reaction was undertaken for 3 cycles at 94°C (30 s), 50°C (30 s), and 72°C (30 s) followed by 30 cycles at 94°C (30 s), 55°C (30 s) and 72°C (30 s). This generated a 412-bp long fragment containing a *Xho*I site at its right end and a *Nde*I site at its left end. The fragment was gel purified, digested with *Xho*I and *Nde*I, and ligated between the *Xho*I and *Nde*I sites of pLTD1D, resulting in a 984-bp long PR-1 promoter fragment fused to GUS.

D. Construction of pLTD7D

Plasmid pLTD1D was digested with restriction enzymes *Xho*I and *Bst*EII; the protruding ends of both *Xho*I and *Bst*EII sites were filled-in with Klenow DNA polymerase; and the vector fragment containing a 815-bp long promoter fragment was self-ligated.

E. Construction of pLTD71D

Plasmid pLTD1D was used as template for PCR with a left-to-right "topstrand" primer comprising a *Xho*I restriction site and extending from position -733 to -714 upstream of the PR-1 ATG (primer N781: ACC GCT CGA GAT AAA TCT CAA TGG GTG ATC) (SEQ ID NO: 9) and a right-to-left "bottomstrand" primer extending from position -584 to -607 upstream of the PR-1 ATG (primer PR1R: TAT TTG TTT CTT AGT GTT TCA TGC) (SEQ ID NO: 8). The PCR reaction was undertaken for 3 cycles at 94°C (30 s), 50°C (30 s), and 72°C (30 s) followed by 30 cycles at 94°C (30 s), 55°C (30 s) and 72°C (30 s). This generated a 126-bp long fragment containing a *Xho*I site at its right end and a *Nde*I site at its left end. The fragment was gel purified, digested with *Xho*I and *Nde*I, and ligated between the *Xho*I and *Nde*I sites of pLTD1D, resulting in a 698-bp long PR-1 promoter fragment fused to GUS.

F. Construction of pLTD72D

Plasmid pLTD1D was used as template for PCR with a left-to-right "topstrand" primer comprising a *Xho*I restriction site and extending from position -656 to -637 upstream of the PR-1 ATG (primer N704: ACC GCT CGA GTT CTT CAG GAC TTT TCA GCC) (SEQ ID NO: 10) and a right-to-left "bottomstrand" primer extending from position -584 to -607 upstream of the PR-1 ATG (primer PR1R: TAT TTG TTT CTT AGT GTT TCA TGC) (SEQ ID NO: 8). The PCR reaction was undertaken for 3 cycles at 94°C (30 s), 50°C (30 s), and 72°C (30 s) followed by 30 cycles at 94°C (30 s), 55°C (30 s) and 72°C (30 s). This generated a 49 bp long fragment containing a *Xho*I site at its right end and a *Nde*I site at its left end. The fragment was gel purified, digested with *Xho*I and *Nde*I, and ligated between the *Xho*I and *Nde*I sites of pLTD1D, resulting in a 621-bp long PR-1 promoter fragment fused to GUS.

G. Construction of pLTD8D

Plasmid pLTD1D was digested with restriction enzymes XhoI and NdeI; the protruding ends of both *Xho*I and *Nde*I sites were filled-in with Klenow DNA Polymerase; and the vector fragment containing a 572-bp long promoter fragment was self-ligated.

H. Construction of pLTD9D

Plasmid pLTD1D was digested with restriction enzymes *Xho*I and *Bgl*II; the protruding ends of both *Xho*I and *Bgl*II sites were filled-in with Klenow DNA Polymerase; and the vector fragment containing a 78-bp long promoter fragment was self-ligated.

Example 4: Plant Transformation

A. Construction of Binary Vectors

Plasmid pLTD1D was digested with restriction enzymes *Xho*I and *Sac*I releasing a 6,422-bp long fragment that was gel purified and inserted between the *Sal*I and *Sac*I sites of pCIB200 (U.S. Patent No. 5,614,395), resulting in pCIB/LTD1D. Plasmids pLTD5D, pLTD6D, pLTD61D, pLTD7D, pLTD71D, pLT72D, pLTD8D, and pLTD9D were digested with restriction enzymes *Kpn*I and *Sac*I. The resulting PR-1 promoter-GUS gene fusions (4,138-bp, 3,457-bp, 3,148-bp, 2,979-bp, 2,862-bp, 2,785-bp, 2,736-bp and 2,242-bp long fragments, respectively) were gel purified and inserted between the *Kpn*I and *Sac*I sites of pCIB200, resulting in plasmids pCIB/LTD5D, pCIB/LTD6D, pCIB/LTD61D, pCIB/LTD7D, pCIB/LTD71D, pCIB/LTD72D, pCIB/LTD8D, and pCIB/LTD9D, respectively.

B. Transformation of Arabidopsis

The binary vector constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (Bechtold, N. *et al.*, CR Acad. Sci. Paris, Sciences de la vie, 316:1194-1199 (1993)) by electroporation (Dower, W.J., Mol. Biol. Rep 1:5 (1987)). Arabidopsis was transformed using the vacuum infiltration method (Bechtold, N. *et al.*, CR Acad. Sci. Paris, Sciences de la vie, 316:1194-1199 (1993)). Seeds obtained from infiltrated plants (T1 seeds) were plated on 50mg/l of kanamycin sulfate and resistant T1 plants were transfered to soil.

C. Transformation of Maize

The binary vector constructs are transformed into maize using the method described by Koziel *et al.*, Biotechnology 11: 194-200, (1993) using particle bombardment into cells of immature embryos.

D. Transformation of Wheat

The binary vector constructs are transformed into immature wheat embryos and immature embryo-derived callus using particle bombardment as described by Vasil *et al.*,

Biotechnology 11: 1553-1558 (1993), and Weeks *et al.*, Plant Physiology 102: 1077-1084 (1993).

Example 5: Determination of the Inducibility of Gene Expression by Chemical Regulators

A. Treatment with INA

For each transgenic line, seeds obtained from T1 plants were harvested (T2 seeds) and plated on duplicate plates containing 50 mg/l of kanamycin sulfate. After twenty days, one plate was treated by spraying with 0.25 mg/ml INA while the duplicate was kept as a control. Three days later, green tissue was harvested, flash frozen, and kept at -70°C .

B. GUS Enzyme Assay

Frozen tissue was homogenised to a fine powder under liquid nitrogen. Extracts were prepared in GUS assay buffer (50 mM sodium phosphate pH 7.0, 0.1% Triton-X 100, 0.1% sarkosyl, 10 mM beta-mercaptoethanol) as described by Jefferson, R.A. *et al.*, *Proc. Natl. Acad. Sci.* USA 83, 8447-8451 (1986). The reactions were carried out in the wells of microtiter plates by mixing 10 µl of extract with 65 µl of GUS assay buffer containing 4-methyl umbelliferyl glucuronide (MUG) at a final concentration of 2 mM in a total volume of 75 µl. The plates were incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 225 µl of 0.2 M sodium carbonate. The concentration of fluorescent indicator released was determined by reading the plates on a Flow Labs Fluoroskan II ELISA plate reader. Duplicate fluorescence values for each samples were averaged, and background fluorescence (reaction without MUG) was substracted to obtain the concentration of MU for each sample. The amount of protein in each extract was determined in a Bradford assay (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's instructions. The specific activity was determined for each sample and was expressed in pmoles MU/mg protein/minute.

Table 1 shows the average values of GUS activity (INA-treated/untreated controls) for the transgenic lines containing the PR-1 promoter constructs. Here, GUS values are expressed in pmole MU/mg protein/min, and the number of independent transgenic lines used for the determination of each value are shown in column (N). For each independent transgenic line, the induction of GUS expression by INA was obtained by dividing the

specific activity of the INA-treated sample by the specific activity of the untreated control sample.

Table 1

Promoter	Control	INA-Treated	Fold Induction	N
4,258-bp	183	1,076	35	18
1,974-bp	113	1,019	18	22
1,293-bp	106	955	10	21
984-bp	36	398	13	14
815-bp	99	1,064	19	20
698-bp	78	314	5	19
621-bp	57	52	0.9	19
572-bp	165	183	1.1	19
78-bp	143	137	1.2	19

As shown in Table 1, upon treatment with INA, plants transformed with the chimeric gene including the full-length (4,258-bp long) PR-1 promoter demonstrated greatly increased induction of GUS expression compared to controls. In addition, the 1,974-bp, 1,293-bp, 984-bp, and 815-bp long promoter fragments yielded similar induction of GUS expression by INA. The 698-bp long promoter fragment still yielded high inducibility by INA, although reduced by approximately 3-4 fold compared to the longer promoter fragments. However, the 621-bp, 572-bp, and 78-bp long promoter fragments yielded substantially no induction of GUS expression by INA.

C. Treatment with BTH

Instead of INA as described above in Example 5A, plant material is sprayed with BTH as described by Goerlach *et al.*, *The Plant Cell* 8: 629-643, (1996); Friedrich *et al.*, *Plant Journal* (1996); and Lawton *et al.*, *Plant Journal* (1996). BTH treated plant tissue is then subjected to a GUS assay as described above in Example 5B.

D. Treatment with salicylic acid (SA)

Instead of INA as described above in Example 5A, plant material is sprayed with 5mM SA, sodium salt. SA treated plant tissue is then subjected to a GUS assay as described above in Example 5B.

C. Linker-Scanning Mutant Analysis

Example 6: Preparation of Chimeric Genes Containing Linker-Scanning Mutants of the *Arabidopsis* PR-1 Promoter Sequence Fused to the β-glucuronidase (GUS) Reporter Gene

A. Contruction of pLS1 to pLS9

Plasmid pLTD6D was used as a template in PCR with a left-to-right "topstrand" primer extending from positions -887 to -867 (primer Anc1: AGG TAT ACT GGA GAT AGG AGG) upstream of the PR-1 ATG and a right-to-left "bottomstrand" primer comprising 26-bp of PR-1 promoter sequence (positions -715 to -741 upstream of the PR-1 ATG) and a further 10-bp containing a XbaI restriction site (primer LS1-: GCT CTA GAG GGA AAA AAA AAA AAA AAA AAA AAA AAA (SEQ ID NO:11)). This PCR reaction was undertaken with AmpliTaq DNA polymerase according to the manufacturer's recommendations (Perkin Elmer/Roche, Branchburg, NJ) for three cycles at 94°C (30 s), 50°C (30 s), and 72°C (30 s), followed by 30 cycles at 94°C (30 s), 55°C (30 s) and 72°C (30 s). This generated a product of 184-bp (fragment A1) through annealing of the homologous PR-1 promoter sequences; the fragment included a BstEII site from the PR-1 promoter at its left end and a XbaI site at its right end. A second PCR reaction was done using the same template with a left-to-right "topstrand" primer comprising 22-bp of PR-1 promoter sequence (positions -730 to -708 upstream of the PR-1 ATG) and a further 10-bp containing a XbaI restriction site (primer LS1+: GCT CTA GAG CAA TCT CAA TGG GTG ATC TAT TG (SEQ ID NO:12)) and a right-to-left "bottom strand" primer extending from positions -584 to -607 upstream of the PR-1 ATG (primer PR1R: TAT TTG TTT CTT AGT GTT TCA TGC (SEQ ID NO:8)). This PCR reaction was done under the same conditions as the one described above and generated a fragment of 194-bp (fragment B1) through annealing of the homologous PR-1 promoter sequences; this fragment included a XbaI site at its left end and a NdeI site from the PR-1 promoter at its right end.

Fragments A1 and B1 generated above were gel purified using standard procedures to remove oligonucleotides. Fragment A1 was cleaved with BstEII and XbaI (all restriction enzymes were purchased from Promega, Madison, WI) and fragment B1 was cleaved with XbaI and NdeI. Both fragments were ligated into plasmid pLTD6D that had previously been digested with restriction enzymes BstEII and NdeI, resulting in plasmid pLS1.

Plasmids pLS2 to pLS9 were constructed using the same strategy. For each construct a fragment A (A2 to A9) was amplified using left-to-right "topstrand" primer Anc1 and a "bottomstrand" primer (LS2- to LS9-, respectively) and a fragment B (B2 to B9) was amplified using a left-to-right "topstrand" primer (LS2+ to LS9+, respectively) and "bottomstrand" primer PR1R. PCR fragments were gel purified and digested with the appropriate restriction enzymes (BstEII and XbaI for fragments A, XbaI and NdeI for fragments B). Corresponding pairs were ligated into pLTD6D as described before, resulting in plasmids pLS2 to pLS9.

primer LS2-: GTC CTA GAG CTA TCC AAA AAG AAA AAA AAA AAA A (SEQ ID NO:13)

primer LS3-: GTC CTA GAT ACA TTG AGA TTT ATC CAA AAA G (SEQ ID NO:14)

primer LS4-: GTC CTA GAA TAT AGA TCA CCC ATT GAG ATT T (SEQ ID NO:15)

primer LS5-: GTC CTA GAT TGA AAC AGT CAA TAG ATC ACC (SEQ ID NO:16)

primer LS6-: GTC CTA GAG GGT GAC GTA GAG AAA CAG TCA A (SEQ ID NO:17)

primer LS7-: GTC CTA GAA AAA GTA AAA TAG TGA CGT AGA G (SEQ ID NO:18)

primer LS8-: GTC CTA GAT TTC TAT GAC GTA AGT AAA ATA GTG (SEQ ID NO:19)

primer LS9-: GTC CTA GAC GTG CCG CCA CAT CTA TGA CGT A (SEQ ID NO:20)

primer LS2+: GTC CTA GAA TGG TGA TCT ATT GAC TGT TTC TC (SEQ ID NO:21)

primer LS3+: GTC CTA GAG ATG ACT GTT TCT CTA CGT CAC (SEQ ID NO:22)

primer LS4+: GTC CTA GAA TTC TAC GTC ACT ATT TTA CTT AC (SEQ ID NO:23)

primer LS5+: GTC CTA GAT ATA TTT TAC TTA CGT CAT AGA TGT G (SEQ ID NO:24)

primer LS6+: GTC CTA GAG AAC GTC ATA GAT GTG GCG GCA (SEQ ID NO:25)

primer LS7+: GTC CTA GAT GTG TGG CGG CAT ATA TTC TTC AG (SEQ ID NO:26)

primer LS8+: GTC CTA GAT TTA TAT TCT TCA GGA CTT TTC AGC (SEQ ID NO:27)

primer LS9+: GTC CTA GAA TAG GAC TTT TCA GCC ATA GGC (SEQ ID NO:28)

B. Construction of pLS10 to pLS12

For each construct, a fragment A (A10 to A12) was produced as described above using left-to-right "topstrand" primer Anc1 and a right-to-left "bottomstrand" primer (LS10- to

LS12-, respectively). However, instead of producing a corresponding fragment B, a pair of complementary oligonucleotides (LS102 and LS103 for pLS10, LS112 and LS113 for pLS11, LS122 and LS123 for pLS12) comprising PR-1 promoter sequences and the desired mutation in the PR-1 promoter were used. Each pair of annealed complementary oligonucleotides contained a XbaI overhang at its left end and a NdeI overhang at its right end. PCR fragments were gel purified and digested with BstEII and XbaI. Corresponding fragments A and annealed complementary oligonucleotides were ligated into pLTD6D as described before, resulting in plasmids pLS10 to pLS12.

primer LS10-: GTC CTA GAA CGA AGA ATA TAT GCC GCC AC (SEQ ID NO:29)
primer LS11-: GTC CTA GAA GGA AAA GTC CTG AAG AAT ATA TG (SEQ ID NO:30)
primer LS12-: GTC CTA GAA AGC CTA TGG CTG AAA AGT CC (SEQ ID NO:31)
primer LS102: CTA GAG GAG CCA TAG GCA AGA GTG ATA GAG ATA CTC A (SEQ ID NO:32)
primer LS103: TAT GAG TAT CTC TAT CAC TCT TGC CTA TGG CTC CT (SEQ ID NO:33)
primer LS112: CTA GAT GAA GAG TGA TAG AGA TAC TCA (SEQ ID NO:34)
primer LS113: TAT GAG TAT CTC TAT CAC TCT TCA T (SEQ ID NO:35)
primer LS122: CTA GAT AGA GAT ACT CA (SEQ ID NO:36)
primer LS123: TAT GAG TAT CTC TAT (SEQ ID NO:37)

C. Construction of pLS13

A PCR fragment was produced as described above using left-to-right "topstrand" primer AncI and a right-to-left "bottomstrand" primer comprising 24-bp of PR-1 promoter sequence (positions -618 to -642 upstream from the PR-1 ATG), the desired mutation in PR-1 promoter and a NdeI restriction site (primer LS13-: GGA ATT CCA TAT GCC AGA AGT CTT CAC TCT TGC CTA TGG CTG AAA AG (SEQ ID NO:38)). The resulting 282-bp long fragment was gel purified, digested with BstEII and NdeI and ligated into pLTD6D as described before, resulting in pLS13.

Example 7: Transformation of Arabidopsis

A. Construction of pCIB200

TJS75Kan was first created by digestion of pTJS75 (Schmidhauser and Helinski, J. Bacteriol. 164: 446-455 (1985)) with NarI to excise the tetracycline gene, followed by insertion of an AccI fragment from pUC4K (Messing, J. and Vierra, J., Gene 19: 259-268 (1982)) carrying a NptI gene. pCIB 200 was then made by ligating XhoI linkers to the EcoRV fragment of pCIB7 (containing the left and right T-DNA borders, a plant selectable nos/nptII chimeric gene and the pUC polylinker; Rothstein, S.J. et al., Gene 53: 153-161 (1987)) and cloning XhoI digested fragment into SalI digested TJS75Kan.

B. Construction of binary vectors

Plasmids pLS1 to pLS13 were digested with restriction enzymes KpnI and SacI. The fragments containing the PR-1 promoter-GUS fusions were gel purified and ligated between the KpnI and SacI sites of pCIB200, resulting in plasmids pCIB/LS1 to pCIB/LS13, respectively.

C. Vacuum infiltration of Arabidopsis

The binary vector constructs described in this example were transformed into Agrobacterium tumefaciens strain GV3101 (Berchtold, N. et al., CR Acad. Sci. Paris, Sciences de la vie, 316:1194-1199 (1993)) by electroporation (Dower, W.J., Mol. Biol. Rep 1:5. (1987)). Arabidopsis was transformed using the vacuum infiltration method (Berchtold, N. et al., CR Acad. Sci. Paris, Sciences de la vie, 316:1194-1199 (1993)). T1 seeds were plated on 50mg/l of kanamycin sulfate and resistant transformed lines were transfered to soil.

D. Transformation of Maize

The binary vector constructs described in this example are transformed into maize using the method described by Koziel *et al.*, Biotechnology 11: 194-200, (1993) using particle bombardment into cells of immature embryos.

E. Transformation of Wheat

The binary vector constructs described in this example are transformed into immature wheat embryos and immature embryo-derived callus using particle bombardment as described by Vasil *et al.*, Biotechnology 11: 1553

Example 8: Determination of the Inducibility of GUS expression by Chemical Regulators

A. Treatment with INA

T2 seeds of each transformed line were harvested and plated on duplicate plates containing 50mg/l of kanamycin sulfate. After twenty days, one plate for each independent transgenic line was treated by spraying with 0.25 mg/ml INA (isonicotinic acid) while the duplicate was kept as control. Three days later, the seedlings were harvested, deep frozen and kept at -70°C.

B. Beta-Glucuronidase (GUS) Enzyme Assay

Frozen leaf tissue was ground in a mortar with a pestle in the presence of liquid nitrogen to produce a fine powder. Leaf extracts are prepared in GUS extraction buffer (50 mM sodium phosphate pH7.0, 0.1% Triton-X 100, 0.1% sarkosyl, 10 mM beta-mercaptoethanol) as described by Jefferson, R.A. et al., PNAS USA 83, 8447-8451 (1986). The reactions are carried out in the wells of microtiter plates by mixing 10 µl of extract with 65 µl of GUS assay buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 10 mM beta-mercaptoethanol) containing 4-methyl umbelliferyl glucuronide (MU) at a final concentration of 2mM in a total volume of 75 µl. The plate was incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 225 µl of 0.2 M sodium carbonate. The concentration of fluorescent indicator released was determined by reading the plate on a Flow Labs Fluoroskan II ELISA plate reader. Duplicate fluorescence values for each samples were averaged, and background fluorescence (reaction without MUG) was substracted to obtain the concentration of MU for each sample. The amount of protein in each extract was determined using the Bio-Rad Protein Assay (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's recommendations. The specific activity was determined for each sample and was expressed in pmoles MU/mg protein/minute.

Table 2 shows the average values of GUS activity (INA-treated/untreated controls) for the transgenic lines containing the linker-scanning mutant promoter constructs. Here, GUS values are expressed in pmole MU/mg protein/min, and the number of independent transgenic lines used for the determination of each value are shown in column (N). For each independent transgenic line, the induction of GUS expression by INA was obtained by dividing the specific activity of the INA-treated sample by the specific activity of the untreated control sample.

Table 2

Construct	Control	INA-Treated	Fold Induction	N
LS1	44	250	5.9	20
LS2	69	658	9.8	20
LS3	76	676	10.4	20
LS4	316	4,073	20.8	20
LS5	302	1,202	6.0	20
LS6	194	623	4.5	20
LS7	104	137	1.4	20
LS8	58	640	12.8	19
LS9	169	931	4.7	20
LS10	207	237	1.7	30
LS11	270	603	4.8	19
LS12	96	611	11.8	20
LS13	128	535	5.3	20
wt	107	997	11.6	21

As shown in Table 2, most LS mutations had no effect or minor effects on the promoter activity. However, 3 of them had dramatic effects on the promoter function. One construct, LS4 (introducing a mutation at positions -666 to -675 (nucleotides 3584 to 3593 of SEQ ID NO: 1)), resulted in 2-fold higher average induction of GUS activity by INA than the control construct and approximatively 4-fold and 3-fold higher average GUS activity for INA- and water-treated samples, respectively. This suggested that a negative regulatory element had been mutated in this construct. Since both induced and uninduced expression levels were influenced, the mutation seemed to affect a constitutive negative regulatory element. In two constructs, LS7 (mutation at positions -636 to -645 (nucleotides 3614 to 3623 of SEQ ID NO: 1)) and LS10 (mutation at positions -606 to -615 (nucleotides 3644 to 3653 of SEQ ID NO: 1)), a complete loss of inducibility of GUS activity by INA was observed. Average GUS values for both water and INA treatments in transgenic lines containing LS7 were similar to water-treated values for lines containing the control construct. In the case of LS10, average GUS values were slightly higher because of two lines showing high uninduced and induced GUS activity. These results are consistent with the presence of a positive regulatory element that is necessary for induction of PR-1 gene expression by INA in or near the LS7 and LS10 locations.

C. Treatment with BTH

Instead of INA as described above in Example 8A, plant material is sprayed with BTH as described by Goerlach *et al.*, *The Plant Cell* 8: 629-643, (1996); Friedrich *et al.*, *Plant*

Journal (1996); and Lawton *et al.*, *Plant Journal* (1996). BTH treated plant tissue is then subjected a GUS assay as described above in Example 8B.

D. Treatment with salicylic acid (SA)

Instead of INA as described above in Example 8A, plant material is sprayed with 5mM SA, sodium salt. SA treated plant tissue is then subjected a GUS assay as described above in Example 8B.

D. *In-vivo* Footprinting Analysis

Example 9: *In-vivo* Footprinting

Arabidopsis plants (ecotype Columbia, Lehle Seeds, Tucson, AZ) were grown in autoclaved Fafard super-fine germinating mix in growth chambers with 60% humidity for 9 hours at ~250 pmol photon/m2/s and 20°C and for 15 hours in the dark at 18°C. After three to four weeks, they were sprayed with 0.65mM INA. At different time intervals, plants were vacuum infiltrated in MS salts containing 0.04 to 1% DMS and 0.01% Silwet L-77 (Osi Specialties) for 2 min at room temperature, washed twice in ice-cold water, flash frozen in liquid nitrogen and lyophilised for 2 days. DNA was isolated with a modified CTAB method. Lyophilised material was extracted in 100mM Tris-HCl pH 7.5, 1% CTAB, 0.7M NaCl, 10mM EDTA, 1% b-mercaptoethanol at 60°C for 45 min. After a chloroform/isoamylalcohol extraction, 0.6 volume isopropanol was added to the aqueous phase and incubated for 30 min at room temperature. The precipitate was resuspended in TE buffer and treated with RNaseA for 30 min at 37°C. DNA was reprecipitated with 0.1 volume sodium acetate and 2.5 volumes ethanol and resupended in TE buffer. DNA was cleaved with 1M piperidine for 30 min at 90°C, lyophilised 3 times and resuspended in dionised water. As a control, purified genomic DNA was treated with 0.5% DMS for 30 sec at room temperature and cleaved with piperidine as described above.

Ligation-mediated PCR (LM-PCR) was carried out (Mueller, P.R. and Wold, B, Science 246:780-786 (1989) and Brignon, P. et al, Plant Molecular Biology Manual B18:1-34 (1993)). For analysis of the coding strand, piperidine-cleaved DNA and primer P1- (ATT TAC AGT CAG AAA AAA TAA AAG, positions -479 to -503 (SEQ ID NO: 39)) were heated at 95°C for 5 min, annealed at 50°C for 30 min and first strand synthesis was carried

out at 42°C for 5 min with Sequenase Version 1.0 (USB Biochemicals). A unidirectional staggered linker formed by LMPCR2 (GAA TTC AGA TC (SEQ ID NO: 40)) and LMPCR3 (TGA CCC GGG AGA TCT GAA TTC (SEQ ID NO: 41)) was ligated to the blunt-ended DNA molecules for 15 hours at 17°C. Exponential PCR was carried out with primers LMPCR3 and P2- (AGT TTA TAT CTA CAG TCA ATT TTC AAA, -502 to -529 (SEQ ID NO: 42)) using KCl-based Taq poymerase buffer supplemented with 2.5mM MgCl2 under following conditions: 16 cycles at 94°C/1min, 55°C/2 min, 74°C/3 min and an addition of 5 sec to the extension step for every cycle, followed by one cycle at 94°C/1min, 55°C/2 min, 74°C/10 min. The end-labeling PCR was carried out using primer P3- (GTT TAT ATC TAC AGT CAA TTT TCA AAT AAA AG, -503 to -535 (SEQ ID NO: 43)) in 5 cycles at 94°C/1min, 60°C/2 min, 76°C/3 min. Non-coding strand analysis was carried out similarly using primer P41+ (CTT TTT TTT TTT TTT TTT TTT TTT TTT TC, -735 to -706 (SEQ ID NO: 44)) for first strand synthesis annealed to plant DNA at 50°C. A unidirectional staggered linker formed by LMPCR2 (GAA TTC AGA TC (SEQ ID NO: 40)) and LMPCR1 (AGT TAC TAG TGA GAT CTG AAT TC (SEQ ID NO: 45)) was ligated to the blunt-ended DNA molecules and exponential PCR was carried out with primers LMPCR1 and P52+ (TTT TTT TTT TTT TTT CTT TTT GGA TAA ATC, -722 to -692 (SEQ ID NO: 46)) using an annealing temperature of 55°C. End-labelling PCR was done with primer P53+ (TTT TTT TTT TTT TTT CTT TTT GGA TAA ATC TC, -722 to -690 (SEQ ID NO: 47)) using an annealing tempetrature of 60°C. Amplified fragments were separated on a 0.4mm thick, 6% polyacrylamide gel and dried for 30 min at 80°C. BioMax MR films were used without intensifying screen.

Analysis of the coding strand revealed inducible footprints at positions -629 and -628 and at position -604 (FIG.1) (nucleotides 3630, 3631, and 3655 of SEQ ID NO: 1, respectively). At both sites, an increased band intensity after INA treatment was detected, indicating deprotection of these guanines upon INA treatment. These two footprints are located in sequences mutated by linker-scanning constructs LS8 and LS11 (FIG. 1). Neither construct seemed to significantly influence the regulation of the promoter, but they are surrounding the sequence mutated in construct LS10, which had lost inducibility by INA. This suggests that that the region of the PR-1 promoter around this site undergoes changes in its occupancy by DNA-binding proteins. No other change in band intensities could be

detected in the range of DMS concentrations tested (0.04% to 1%), suggesting that the described sites are the only ones affected after INA treatment.

Examination of the non-coding strand revealed an inducible footprint at position -641 (3618 of SEQ ID NO: 1). In this case, too, deprotection was observed. The guanine at position -641 is included in the sequence mutated in linker-scanning mutant LS7, which had lost inducibility by INA and that contains a recognition site for basic leucine zipper transcription factors at positions -645 to -640. Different DMS concentrations did not reveal any other inducible footprint.

These results show that the examined region undergoes changes in protein-DNA interactions and suggest that the above described elements are required for the induction itself ("switch" element) and are not just binding sites for constitutive transcriptional activators.

E. Methods for Isolating Transcriptional Regulatory Proteins

Example 10: Screening of Expression Libraries (South-Western)

An oligomer containing defined parts of the PR-1 promoter, such as the sequence of LS4, LS7, or LS10, is used to screen a cDNA expression library (Singh, H. et al. Biotechniques 7:252-261 (1989). A cDNA expression library is plated and the proteins are transferred onto a nitrocellulose filter. The filter is probed with a radiolabelled oligomer containing one or more copies of the sequence of interest. Clones expressing proteins that bind to this sequence are detected by autoradiography and isolated.

Example 11: Yeast One-Hybrid System

An oligomer containing defined parts of the PR-1 promoter, such as the sequence of LS4, LS7, or LS10, is used as a bait in a yeast one-hybrid system (Li, J.J. and Herskowitz, I. Science 262: 1870-1874 (1993)). The chosen sequence is fused upstream of a minimal promoter and a reporter gene and transformed into yeast. The resulting yeast strain is transformed with a cDNA expression library fused to a yeast activation domain. Upon specific interaction between the bait and a fusion protein, transcription of the reporter gene is activated. The corresponding clone is isolated.

F. Methods for Construction of Inducible Hybrid Promoters

Example 12: Inducible Hybrid Promoters

The above described elements (one repeat or preferably several repeats) are fused to a minimal promoter in order to obtain inducible gene expression. For example, the region of the PR-1 promoter spanning LS7 through LS10 (nucleotides 3614-3653 of SEQ ID NO: 1) may be used to confer inducibility to a promoter fragment. Transcriptional enhancer elements are also included into the synthetic promoter in order to obtain increased gene expression.

Example 13: Inducible Tissue- or Organ-Specific Promoters

The elements described above in Example 12 (one repeat or preferably several repeats) are fused to or included into promoters that confer tissue- or organ-specific gene expression in order to obtain inducible gene expression in a particular tissue or organ.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lebel, Edouard
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Thorne, Leigh
Uknes, Scott
Ward, Eric

(ii) TITLE OF INVENTION: Chemically Inducible Arabidopsis PR-1 Promoter

(iii) NUMBER OF SEQUENCES: 51

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4505 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: promoter
(B) LOCATION: 1..4258
(D) OTHER INFORMATION: /note= "Full-length Arabidopsis PR-1 Promoter Sequence"

(ix) FEATURE:
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(B) LOCATION: 4229..4232

(ix) FEATURE:
(A) NAME/KEY: misc_signal
(B) LOCATION: 4294..4296
(D) OTHER INFORMATION: /note= "Start codon for translation"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2966..4258
(D) OTHER INFORMATION: /note= "1293-bp long Arabidopsis thaliana PR-1 promoter fragment in plasmid pLTD6D - used for LS construct construction"

(ix) FEATURE:
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(B) LOCATION: 3444..4258
(D) OTHER INFORMATION: /note= "815-bp long Arabidopsis thaliana PR-1 promoter fragment in plasmid pLTD7D"

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(B) LOCATION: 3561..4258
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(ix) FEATURE:
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(B) LOCATION: 3638..4258
(D) OTHER INFORMATION: /note= "621-bp long Arabidopsis thaliana PR-1 promoter fragment in plasmid pLTD72D"

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(B) LOCATION: 3554..3563
(D) OTHER INFORMATION: /note= "LS1"

(ix) FEATURE:
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(B) LOCATION: 3564..3573
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(B) LOCATION: 3614..3623

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(B) LOCATION: 3624..3633
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(A) NAME/KEY: misc_feature
(B) LOCATION: 3634..3643
(D) OTHER INFORMATION: /note= "LS9"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3644..3653
(D) OTHER INFORMATION: /note= "LS10"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3654..3663
(D) OTHER INFORMATION: /note= "LS11"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3664..3671
(D) OTHER INFORMATION: /note= "LS12"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3672..3681
(D) OTHER INFORMATION: /note= "LS13"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GTATTGTAAA AGAATTATGC AAGCGCAACC TACATGACTA GTGGAGATAT CATATGACTA 180

CTTATCAGGG TTGGTGAATT TTAAATATTC CCAGACTGCT GCTAAAACCT TAATGCATCC 240

AATTGGTGTT GACGAGATGG CTATCCTGCT CAGTTTTTAA TTGCTGCTTG GTCGATCTTG 300

GAAATGATTT TATAATAGCA GTGCAGTCCT TCTTGATCTT TGGATGCATG CCCAAAAGTG 360

TTAAATCAAC TATTCTGACT CTTGTTCCCA AAACAGATGG TGCACAAAAT ATGAAGGAGT 420

TCATACTGAT AGCGTGATGC AATCTTCTAT ATTAAGTGAT ATATAAGATA ATAGCAAACC 480

GGCTTAAAGT TACTTTACAA GAGGCGATGG AACCGAATCA GAGCACCTTT GTGAAGGGGA 540

GGCTCTTACT AGAGAACATA TTTTTAGCAA CAAAACTAGT CAAGGACTAC CACAAGCAAT 600

CACTCTCATC TCGTTTAGCA ATTAAGCTTG ATATCTCTAA AGCGTTTGAC ATAGAGCAAT 660

GGCCGTTTAT TGCTGCTAGG CTACGTGTGA TGGGTTATCC ATAGCTCTTT ATACACTAGA 720

TAAATATATG CATCTCTACG TCCTCGTTTT GTTTTTTTCT CTAGCTCTTG TGGTATAAGG 780

AAAGGATGCT CTCTTTCACC GTACTTCTAT GTTATCATCA ACAATGTTTT GTCGACTATG 840

TTAAACAGAG CAGCTGTTAT GAAAGAGATT GGTTCTCACC CGTTTTGCAA GGAGATAAAG 900

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CAAAAGCAAT GACGAGACTT GACTACAAAC CTCTACTTGA CAAGATAAGG TCTCGTTTTT 1200

TAATTGGACA AGCAAGCACC TCTCACTTGA GGTTGTCTAC AACTTATGAA CTCAGTTATA 1260

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TGAAAGGAGG TGTAGTTCAT TCCTCTAGAG TGGATCATCG CTTGATGCAA CTAAAGCAAA 1380

AGTGTCTTGG GAGGAGGTTT GCTACTCAAA AAAGGAAGGG GGCTTGGGGT TCCGCGTATG 1440

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TCTCTATGGG TGGCATAGAT AAGTCATTAC CTTCTGCGCC AAGAATCATT TTGGGATATC 1560

AAAGCAACGT CCTTAGGGTC TTCGGTTGGA CGTAAGCTGC TCAAGCTTTG CCCACAAGCC 1620

ATTGAGTTTA TAAGAATGGA AGTAAAAGAT GGAGTTAAGA CACGATCCTA GTCGGATACT 1680

TGGTTGTCAA TGGGGAGTCT TATTGATCGT AGTTGGAGAA AGGGGAACAT ATGAATTGGG 1740

AGTGCACCGA GATGCTACAG TTGCAGAGGT TGTAGCAAGA GGTCACTGGT CAATCCGTCG 1800

TGGTCAGAAC CAACATATAA GTTTGATTGT GGACCAGATC ATAGCTAAAG ACCCGTCCGT 1860

ACACTCGGCT AGTCAAGATC ATTGATTAGT ATATATACAT ATTGTATTGC ATGAAAAGTG 1920

TTTAAAGTAA ATTGTGTCCT ATACAAAGAA TATATATAAC GATCATTGAT TAGTATATAT 1980

ACATATTGTA TTGTGTGTTT AAAGTAAATT GTGTCCTATA CAAAGAATAT CTTTGTGGAG 2040

AAGCAAAGAG AATACATACT TACGTAGGAA TCTTTTTGTT TTCTTTTTTC ACAACGTAAG 2100

AATGTTTGCT TCCTTACAAT TCATACTTAT TAACTTACAT ATTATGTTTT CTTTTAAATA 2160

TTAAAAATAA CTAATTTTTA TTAGGCAGCA AGTCATTTAC AAAGTAAAAA ATTTCTCCAT 2220

GCATGTAACC TTCATTTATC ATTCATTTTA GTTTGTAACT TTTTATTAGA TTTTGATCAA 2280

GTTAACCGCT AAAATCTCAT TTTATCCGTT CGCATTAAAG TTAAATAGAT TGCTGACATA 2340

TTTTAAATCT AATAGAAAAT GCCATCTGGC AAATAAACAA CGGACACGAT TTTAAACTAA 2400

ATTTTACCAA AAAGAAAAAA CTTATACGAC TTTTCTTGCT TAGAAGTCTT TGCATTGTTA 2460

ATAGATTGTT GAAAAGGTTT ATTCATTACT TTCATGCAGA GAGATAACAT ATCATCGCGT 2520

GGGGATTTAT TCAATCCAAA GAAAAGCTTC CAAAAACTGA CTCTGCTTCA TGAAACACTC 2580

ACTCTAATTT GCTTCATCAA TCTTAGGACT GACTTTTCCA AWYCAATATG CGGAACTATC 2640

TTCTAATTTA CATTGGTTTC GTGTTTTTTC GAAAGGAGAC AACTATCTTT TTAAAAGCTT 2700

TTCTATAGTG TGATGACAAA AAAAAAATGT AATTGTTAGT TGCAAAAGAA AAGTACAATA 2760

GTCTTTTCTA GTTTTGAGAG TTTAAGGTTT ATGATCGGAA CTTAGAGTKT AAATTTAAAC 2820

TATTTTGTTA ATTTTTGGAC TGATAACAGT TTTTTTTTGA AAATATTGAA ACGTTGTTTA 2880

CCTAATGTAA CATGTTATTC TACTTAAATT ACTTTATATT TTAATAACAT ATAATATTGA 2940

ATAGGATATC ATAGGATATT ATTACGTAAT AATATCCTAT GGTGTCATTT TATAAGTTAG 3000

CACAAGCTTG TTTTAACTTA TAAAATGATT CTCCCTCCAT ATAAAAAAGT TTGATTTTAT 3060

AGAATGTTTA TACCGATTAA AAAAATAATA ATGCTTAGTT ATAAATTACT ATTTATTCAT 3120

GCTAAACTAT TTCTCGTAAC TATTAACCAA TAGTAATTCA TCAAATTTTA AAATTCTCAA 3180

TTAATTGATT CTTGAAATTC ATAACCTTTT AATATTGATT GATAAAAATA TACATAAACT 3240

CAATCTTTTT AATACAAAAA AACTTTAAAA AATCAATTTT TCTGATTCGG AGGGAGTATA 3300

TGTTATTGCT TAGAATCACA GATTCATATC AGGATTGGAA AATTTTAAAG CCAGTGCATA 3360

TCAGTAGTCA AAATTGGTAA ATGATATACG AAGGCGGTAC AAAATTAGGT ATACTGAAGA 3420

TAGAAGAACA CAAAAGTAGA TCGGTCACCT AGAGTTTTTC AATTTAAACT GCGTATTAGT 3480

GTTTGGAAAA AAAAAACAAA GTGTATACAA TGTCAATCGG TGATCTTTTT TTTTTTTTTT 3540

TTTTTTTTTT TTCTTTTTGG ATAAATCTCA ATGGGTGATC TATTGACTGT TTCTCTACGT 3600

CACTATTTTA CTTACGTCAT AGATGTGGCG GCATATATTC TTCAGGACTT TTCAGCCATA 3660

GGCAAGAGTG ATAGAGATAC TCATATGCAT GAAACACTAA GAAACAAATA ATTCTTGACT 3720

TTTTTTCTTT TATTTGAAAA TTGACTGTAG ATATAAACTT TTATTTTTTC TGACTGTAAA 3780

TATAATCTTA ATTGCCAAAC TGTCCGATAC GATTTTTCTG TATTATTTAC AGGAAGATAT 3840

CTTCACAACA TTTTGAATGA AGTAATATAT GAAATTCAAA TTTGAAATAG AAGACTTAAA 3900

TTAGAATCAT GAAGAAAAAA AAACACAAAA CAACTGAATG ACATGAAACA ACTATATACA 3960

ATGTTTCTTA ATAAACTTCA TTTAGGGTAT ACTTACATAT ATACTAAAAA AATATATCAA 4020

CAATGGCAAA GCTACCGATA CGAAACAATA TTAGGAAAAA TGTGTGTAAG GACAAGATTG 4080

ACAAAAAAAT AGTTACGAAA ACAACTTCTA TTCATTTGGA CAATTGCAAT GAATATTACT 4140

AAAATACTCA CACATGGACC ATGTATTTAC AAAAACGTGA GATCTATAGT TAACAAAAAA 4200

AAAAAGAAAA AAATAGTTTT CAAATCTCTA TATAAGCGAT GTTTACGAAC CCCAAAATCA 4260

TAACACAACA ATAACCATTA TCAACTTAGA AAAATGAATT TTACTGGCTA TTCTCGATTT 4320

TTAATCGTCT TTGTAGCTCT TGTAGGTGCT CTTGTTCTTC CCTCGAAAGC TCAAGATAGC 4380

CCACAAGATT ATCTAAGGGT TCACAACCAG GCACGAGGAG CGGTAGGCGT AGGTCCCATG 4440

CAGTGGGACG AGAGGGTTGC AGCCTATGCT CGGAGCTACG CAGAACAACT AAGAGGCAAC 4500

TGCAG 4505

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer ext1"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGAGCACCT ACAAGAGCTA CAAAGACG 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer 1a"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCAAAGCTA CCGATAC 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer 1b"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACGTAACA TTTTTCTAAG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer 1c"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTTAGAAAAA TGTTACGTCC 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer 1d"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTACGCTGCG ATGGATC 17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer N1,076"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCGCTCGAG AATTTTTCTG ATTCGGAGGG 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer PR1R"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TATTTGTTTC TTAGTGTTTC ATGC 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer N781"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACCGCTCGAG ATAAATCTCA ATGGGTGATC 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer N704"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACCGCTCGAG TTCTTCAGGA CTTTTCAGCC 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS1-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCTCTAGAGG GAAAAAAAAA AAAAAAAAAA AAAAAA 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS1+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTCTAGAGC AATCTCAATG GGTGATCTAT TG 32

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS2-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCCTAGAGC TATCCAAAAA GAAAAAAAAA AAAA 34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS3-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCCTAGATA CATTGAGATT TATCCAAAAA G 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS4-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCCTAGAAT ATAGATCACC CATTGAGATT T 31

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS5-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCCTAGATT GAAACAGTCA ATAGATCACC 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS6-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCCTAGAGG GTGACGTAGA GAAACAGTCA A 31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS7-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCCTAGAAA AAGTAAAATA GTGACGTAGA G 31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS8-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTCCTAGATT TCTATGACGT AAGTAAAATA GTG 33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS9-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTCCTAGACG TGCCGCCACA TCTATGACGT A 31

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS2+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTCCTAGAAT GGTGATCTAT TGACTGTTTC TC 32

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS3+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GTCCTAGAGA TGACTGTTTC TCTACGTCAC 30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS4+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCCTAGAAT TCTACGTCAC TATTTTACTT AC 32

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS5+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTCCTAGATA TATTTTACTT ACGTCATAGA TGTG 34

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS6+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTCCTAGAGA ACGTCATAGA TGTGGCGGCA 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS7+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCCTAGATG TGTGGCGGCA TATATTCTTC AG 32

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS8+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTCCTAGATT TATATTCTTC AGGACTTTTC AGC 33

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS9+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTCCTAGAAT AGGACTTTTC AGCCATAGGC 30

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS10-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GTCCTAGAAC GAAGAATATA TGCCGCCAC 29

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS11-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTCCTAGAAG GAAAAGTCCT GAAGAATATA TG 32

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS12-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GTCCTAGAAA GCCTATGGCT GAAAAGTCC 29

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS102"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTAGAGGAGC CATAGGCAAG AGTGATAGAG ATACTCA 37

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS103"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATGAGTATC TCTATCACTC TTGCCTATGG CTCCT 35

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS112"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTAGATGAAG AGTGATAGAG ATACTCA 27

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS113"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATGAGTATC TCTATCACTC TTCAT 25

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS122"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CTAGATAGAG ATACTCA 17

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS123"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TATGAGTATC TCTAT 15

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LS13-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGAATTCCAT ATGCCAGAAG TCTTCACTCT TGCCTATGGC TGAAAAG 47

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer P1-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

ATTTACAGTC AGAAAAAATA AAAG 24

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LMPCR2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GAATTCAGAT C 11

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LMPCR3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TGACCCGGGA GATCTGAATT C 21

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer P2-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AGTTTATATC TACAGTCAAT TTTCAAA 27

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer P3-"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTTTATATCT ACAGTCAATT TTCAAATAAA AG 32

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer P41+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CTTTTTTTTT TTTTTTTTTT TTTTTTTTC 29

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer LMPCR1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AGTTACTAGT GAGATCTGAA TTC 23

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer P52+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTTTTTTTTT TTTTTCTTTT TGGATAAATC 30

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer P53+"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTTTTTTTTT TTTTTCTTTT TGGATAAATC TC 32

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "recognition site of the yeast transcription factor GCN4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TGACTG 6

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = " recognition site of the bZIP transcription factor CREB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CTACGTCA 8

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = " recognition site for bZIP transcription factors"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

ACGTCA 6

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = " consensus recognition sequence of NF-kB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GGGACTTTTC C 11

What Is Claimed Is:

1. An isolated DNA molecule comprising a nucleotide sequence selected from the following group:
   a) a full-length chemically inducible promoter fragment comprising nucleotides 1 through 4258 of SEQ ID NO: 1;
   b) an 815-bp long chemically inducible promoter fragment comprising nucleotides 3444 through 4258 of SEQ ID NO: 1; and
   c) a 698-bp long chemically inducible promoter fragment comprising nucleotides 3561 through 4258 of SEQ ID NO: 1.

2. The isolated DNA molecule of claim 1, comprising the full-length promoter fragment comprising nucleotides 1 through 4258 of SEQ ID NO: 1.

3. The isolated DNA molecule of claim 1, comprising the 815-bp long PR-1 promoter fragment comprising nucleotides 3444 through 4258 of SEQ ID NO: 1.

4. The isolated DNA molecule of claim 1, comprising the 698-bp long PR-1 promoter fragment comprising nucleotides 3561 through 4258 of SEQ ID NO: 1.

5. A chimeric gene comprising the DNA molecule of claim 1 operatively linked to a coding sequence, wherein the DNA molecule regulates transcription of said coding sequence.

6. The chimeric gene of claim 5, wherein said coding sequence encodes an enzyme.

7. The chimeric gene of claim 6, wherein said enzyme is an assayable marker.

8. The chimeric gene of claim 7, wherein said assayable marker is GUS.

9. A recombinant vector comprising the chimeric gene of claim 5.

10. A plant cell stably transformed with the vector of claim 9.

11. The plant cell of claim 10, which is a maize cell.

12. The plant cell of claim 10, which is a wheat cell.

13. The plant cell of claim 10, which is an Arabidopsis cell.

14. An isolated DNA molecule involved in inducibility of a chemically inducible promoter selected from the following group:

a) LS4 comprising nucleotides 3584 through 3593 of SEQ ID NO: 1;

b) LS7 comprising nucleotides 3614 through 3623 of SEQ ID NO: 1;

c) LS10 comprising nucleotides 3644 through 3653 of SEQ ID NO: 1; and

d) a region spanning LS7-LS10 and comprising nucleotides 3614 through 3653 of SEQ ID NO: 1.

15. The isolated DNA molecule of claim 14, wherein said DNA molecule comprises a negative regulatory element and comprises nucleotides 3584 through 3593 of SEQ ID NO: 1.

16. The isolated DNA molecule of claim 14, wherein said DNA molecule comprises an element necessary for inducibility of said chemically inducible promoter and comprises nucleotides 3614 through 3623 of SEQ ID NO: 1.

17. A chemically inducible hybrid promoter comprising the DNA molecule of claim 16 operatively linked to a minimal promoter fragment, whereby said DNA molecule confers inducibility to said minimal promoter fragment.

18. The isolated DNA molecule of claim 14, wherein said DNA molecule comprises an element necessary for inducibility of said chemically inducible promoter and comprises nucleotides 3644 through 3653 of SEQ ID NO: 1.

19. A chemically inducible hybrid promoter comprising the DNA molecule of claim 18 operatively linked to a minimal promoter fragment, whereby said DNA molecule confers inducibility to said minimal promoter fragment.

20. The isolated DNA molecule of claim 14, wherein said DNA molecule comprises elements necessary for inducibility of said chemically inducible promoter and comprises nucleotides 3614 through 3653 of SEQ ID NO: 1.

21. A chemically inducible hybrid promoter comprising the DNA molecule of claim 20 operatively linked to a minimal promoter fragment, whereby said DNA molecule confers inducibility to said minimal promoter fragment.

22. A method of isolating transcriptional regulatory proteins, comprising the steps of:

a) screening a cDNA expression library with an oligomer comprising the DNA molecule of claim 14;

b) detecting clones expressing proteins that bind to said DNA molecule; and

c) isolating said clones expressing said proteins.

23. A method of isolating transcriptional regulatory proteins using a yeast one-hybrid system, comprising the steps of:

a) fusing the DNA molecule of claim 14 upstream of a minimal promoter and a reporter gene coding sequence to construct bait;

b) transforming the bait into yeast;

c) transforming said yeast with a cDNA expression library fused to a yeast activation domain;

d) activating transcription of the reporter gene upon specific interaction between the bait and a transcriptional regulatory protein;

e) detecting a clone expressing said regulatory protein; and

f) isolating said clone expressing said protein.

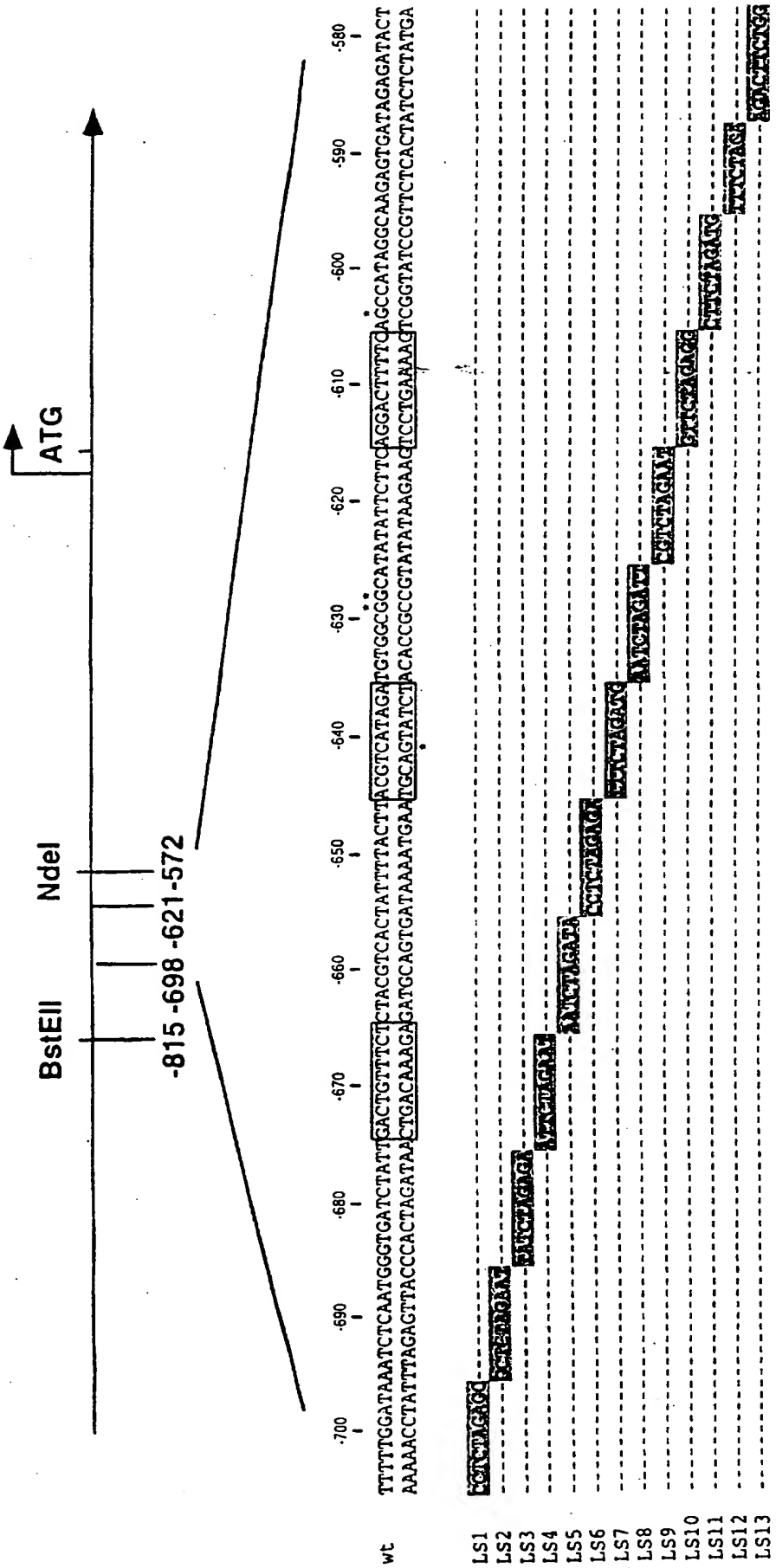
ATG
NdeI
BstEII
-815 -698 -621-572
wt
LS1
LS2
LS3
LS4
LS5
LS6
LS7
LS8
LS9
LS10
LS11
LS12
LS13

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12626

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 5/14, 15/82; C12Q 1/68
US CL :536/23.1, 23.2, 24.1; 435/6, 320.1, 419; 800/205

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.2, 24.1; 435/6, 320.1, 419; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Medline, Biosis, Embase, CAPlus, WPIDS, JAPIO, PATOSEP, PATOSWO; search terms: PR-1, arabidopsis, pathogen related, promoter; sequence search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 95/19443 A2 (CIBA-GEIGY AG) 20 July 1995, pages 31-32, 41-44.	1,2,5-7,9-13 --- 3,4,8,14-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 SEPTEMBER 1997	Date of mailing of the international search report 29 OCT 1997
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Form PCT/ISA/210 (second sheet)(July 1992)*